Migration of a Human Keratinocyte Cell Line (HACAT) to Interstitial Collagen Type I Is Mediated by the $\alpha_2\beta_1$-Integrin Receptor

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The migratory response of the human keratinocyte cell line HaCaT to collagen type I and the molecular mechanism underlying collagen-mediated migration have been analyzed. The migratory response of HaCaT cells to collagen type I consisted of a dose-dependent migration to insoluble step gradients of substratum-bound collagen (haptotaxis) and to gradients of soluble collagen (chemotaxis). Checkerboard analysis demonstrated a minor chemokinetic component. Denatured collagen type I was less chemoattractive than the native triple-helical form. Pre-treatment of cells with 25–250 μg/ml of synthetic peptides containing the fibronectin cell-recognition sequence RGD (Arg-Gly-Asp) resulted in a concentration-dependent inhibition of fibronectin-mediated chemotaxis, whereas chemotaxis to collagen was not affected. We then investigated the role of VLA/collagen-receptors for collagen type I–induced chemotaxis. Monoclonal antibody (MoAb) 5E8, which selectively blocks function of the α_2 subunit of the VLA-2/collagen receptor, dose-dependently inhibited the chemotactic response of HaCaT cells to collagen. This effect was specific for collagen-mediated chemotaxis because the chemotactic response to fibronectin remained unaffected. In contrast, a function blocking MoAb directed to the α_3 subunit of the coexpressed VLA-3 receptor, which is also capable of binding collagen, had no effect. However, function blocking MoAb directed to the β_1-chain of integrins completely inhibited chemotaxis to collagen type I. Based on our results, we propose that the chemotactic migration of the human keratinocyte cell line (HaCaT) to collagen type I is specifically mediated by the RGD independent VLA-2/collagen receptor ($\alpha_2\beta_1$) of the integrin family.
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Epithelial cell migration is a prerequisite for developmental and repair processes such as embryogenesis and epidermal wound healing. Although mechanisms that initiate, coordinate, and finally terminate epithelial cell movements are still far from being fully understood, migration of cells is thought to play a central role. Several agents stimulating cell migration have been characterized for a variety of cells [1–4], among them extracellular matrix proteins [5–10]. Also collagen type I has been found to be chemotactic for fibroblasts and monocytes [8,9]. It is the major newly synthesized structural protein during cutaneous wound healing and following dissolution of the basement membrane collagen type I is directly exposed to epidermal cells. Its stimulatory effect on epidermal cell migration has been discussed previously [11,12]; however, the molecular mechanism underlying this process has not been characterized. In this study we have characterized the chemo- and haptotactic response of a human keratinocyte cell line to collagen type I. Recent studies have revealed a family of cell-surface receptors, termed integrins, that play central roles in mediating cell-substratum interactions [13,14].

The cell-surface heterodimers $\alpha_1\beta_1$ and $\alpha_2\beta_1$, which belong to the integrin superfamily, were initially characterized as "very late antigens" on activated T cells [15] and are now known to function as cell-surface receptors for collagen. Also, the $\alpha_3\beta_1$ integrin has been characterized as a receptor for collagen [16]. In order to investigate the role of the receptors for keratinocyte migration we analyzed their synthesis in HaCaT cells and performed inhibition studies using function-blocking MoAb directed to various α- and β-chains.

Because monoclonal antibodies directed to the α_2- and β_1-chains, but not to the α_3-chain of integrins, dose-dependently inhibited cell migration of the human keratinocyte cell line HaCaT to collagen type I we concluded that the $\alpha_2\beta_1$ receptor is required for the migration of keratinocytes to collagen type I.

MATERIALS AND METHODS

Cell Culture HaCaT cells, a spontaneously transformed non-tumorigenic human keratinocyte cell line with highly preserved phenotypic differentiation characteristics of normal keratinocytes [17–20], were cultivated in DMEM supplemented with penicillin

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Abbreviations:
BSA: bovine serum albumin
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylendiaminetetraacetate
FCS: fetal calf serum
MoAb: monoclonal antibody
NP-40: Nonidet P-40
PBS: phosphate-buffered saline
RGD: arginin-glycine-asparagin tripeptide
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TRIS: trishydroxymethylaminomethane
VLA: very late antigen

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(400 U/ml), streptomycin (50 μg/ml), glutamine (300 μg/ml), and 10% fetal calf serum (FCS) in a humified atmosphere of 5% CO_2 and 95% air at 37°C.

Proteins and Antibodies BSA, RGD peptides, fibronectin, and laminin were purchased from Boehringer Mannheim (Mannheim, FRG), gelatin from BioRad (Munich, FRG), and collagen type I from Biomol (Hamburg, FRG). Purity was verified by SDS-PAGE. The mouse monoclonal antibody (MoAb) 5E8 (IgG1) directed against the α_2 subunit of the $\alpha_2\beta_1$ receptor of integrins was obtained from Dr. Richard Bankert (Roswell Park Memorial Institute, Buffalo, NY) [21,22] and was found to block function of $\alpha_2\beta_1$ (Bankert, unpublished; Hemler et al [23]). The MoAb AIIB2 [24] and 4B4 (IgG1) [25,26] detecting the common β_1 subunit of the VLA subfamily of integrin receptors were kindly provided by Dr. C. Damsky (Departments of Stomatology and Anatomy, University of California, San Francisco, CA). Both MoAb have been found to block the function of the β_1-chain. MoAb J143 (IgG1) [27] directed to the α_3-chain of the VLA-3 receptor was a gift from Dr. L. J. Old (Memorial Sloan Kettering Cancer Center, New York); MoAb P1B5 [16], which also recognize the α_3-chain of the VLA-3, was purchased from Telios Pharmaceuticals Inc. (San Diego, CA). This MoAb interferes with ligand binding to the VLA-3 receptor. MoAb Ts 2/7 [23] detecting the α_1-chain of VLA-1 was kindly provided by Dr. Hemler (Dana Farber Cancer Center, Boston, MA). MoAb GoH_3 was a gift from Dr. Arnoud Sonnenberg (Central Laboratory of the Netherlands, Amsterdam, Holland). This antibody was raised against the α_6 subunit of the $\alpha_6\beta_1$ receptor [28,29] and specifically inhibits the adhesion of platelets to laminin [29].

Several antibodies detecting unrelated surface molecules were used as controls: MoAb W6/32 (IgG2a) directed against a monomorphic determinant of HLA class I molecules; MoAb F19 (IgG1) [30], which recognizes a 95-kDa cell-surface glycoprotein not expressed by HaCaT cells (C. E. Klein, unpublished); and MoAb Lv230 (IgG1) [31], detecting a 130-kDa cell-surface glycoprotein of human keratinocytes were generously provided by Dr. L. J. Old (Memorial Sloan Kettering Cancer Center, New York).

For inhibition studies, hybridoma culture supernatants (GoH_3, AIIB2), diluted ascites of hybridoma-bearing mice (P1B5), or purified MoAb (5E8, 4B4, F19, Lv230, W6/32) were utilized. MoAb had been purified by Protein A affinity chromatography using standard procedures.

Cells of subconfluent cultures were detached by brief exposure to 0.1% trypsin and 0.04% EDTA and were incubated with MoAb at different concentrations ranging from 0.01 to 10 μg/ml in DMEM for 10 min. Hybridoma supernatants were used at different dilutions.

Migration assays were then carried out in the continuous presence of the MoAb in DMEM that had been added to the upper compartment of the Boyden chamber as indicated.

Radioimmunoprecipitation Radioimmunoprecipitation was carried out as previously described [32]. Briefly, cells were metabolically labeled with [^{35}S]-methionine (60–200 μCi/ml) for 16 h. Glycoproteins were isolated from NP-40–solubilized cell extracts by adsorption to Concanavalin A (Con A) (Pharmacia Inc., Uppsala, Sweden). Samples of the Con A–bound fraction (1.5×10^6 cpm) were incubated with 1–2 μg MoAb for 2 h at 4°C. Immune complexes were then precipitated with Protein A–Sepharose CL-4B (Pharmacia, München, FRG) pre-incubated with rabbit anti-mouse IgG (Dako Corp., Santa Barbara, CA). After 10 washes with buffer (0.5% NP-40, 0.1% SDS, 0.15 M sodium chloride, 0.01 M Tris, pH 7.5), precipitated glycoproteins were processed by SDS-PAGE on 9% polyacrylamide gels. For fluorography, gels were immersed in 0.5 M sodium salicylate for 20 min.

Cell Migration Assays

Evaluation of Chemotaxis: HaCaT cell chemotaxis was assayed in triplicate according to Postlethwaite [8] using modified Boyden chambers equipped with polycarbonate filters (13 mm diameter, 8 μm pore size, Nucleopore Corp., Pleasonton, CA). Collagen type I was diluted to the indicated concentrations into serum-free DMEM and added to the lower compartment and the filter was placed above. HaCaT cells were suspended in DMEM without serum or other supplements (2×10^5 cells/ml) and filled into the upper compartment of the chamber (800 μl). The chambers were incubated at 37°C in 5% CO_2, 98% air for 6 h. Then, the cells that had attached to the upper side but had not migrated through the filter were mechanically removed. Consecutively, filters were fixed in 96% ethanol and were stained with Toluidin blue (3% in H_2O) and Diff-Quick (Merz and Dade, Düdingen, Switzerland). Chemotaxis was determined by counting the cells that had migrated to the lower surface of the polycarbonate filters (200-times magnification). For each filter, the number of migrated cells in five randomly chosen microscope fields was determined and the counts were averaged. Random migration was assessed by checkerboard analysis as previously described [1].

Evaluation of Haptotaxis: Haptotaxis was assayed in triplicate using modified Boyden chambers as mentioned above. For these experiments gelatin coating of the filters was necessary. The gelatin-coating procedure was performed as described previously [33] using filters of the same pore size as for the chemotaxis assay. The lower side of the pretreated filter was then coated with collagen by floating the filter overnight on the solution of collagen diluted in DMEM to the concentrations indicated (1–1000 μg/ml), according to the method described by McCarthy, Palm, and Furcht [34]. Untreated filters were used for coating with laminin and fibronectin. As controls, gelatin treated or untreated filters were floated on DMEM alone or on DMEM with BSA (1–1000 μg/ml). Filters to be coated on both sides were submerged overnight in the collagen (or fibronectin or laminin) solution and were then washed five times in PBS and air dried. Chambers were assembled with serum-free DMEM in the lower compartment of the chamber. After addition of 2×10^5 cells to the upper compartment, chambers were incubated in a humified atmosphere at 37°C for 6 h. Filters were then processed and migration determined as for chemotaxis.

Diffusion of Collagen Type I Across the Filter: Experiments were performed to evaluate the diffusion of collagen across the filter during the migration assay. Collagen was labeled with ^{125}I according to the technique of Bolton Hunter as described elsewhere [5]. Specific activity was approximately 4×10^{-2} μCi/mole. Radioactive collagen was added at a concentration of 100 μg/ml to the lower compartment of the Boyden chamber. After assembling chambers, medium was filled into the upper compartment. Samples were sequentially removed from the upper compartment during a 6-h incubation period and radioactivity was counted in a gamma scintillation counter.

Determination of Collagen on Filters: Binding of radioactively labeled collagen type I to gelatin coated or uncoated filters was assessed after their incubation in the chambers (6–12 h) in the absence of HaCaT cells with the lower compartment containing labeled collagen type I (100 μg/ml). The filters were then removed and rinsed twice with PBS. Filter-bound radioactivity was quantitated in a gamma scintillation counter. The amount of collagen bound to the filter was calculated by the following formula:

$$\left(\frac{\text{cpm } ^{125}\text{I on filter}}{\text{cpm total available } ^{125}\text{I}}\right) \times \mu\text{g protein in the lower compartment} = \text{amount protein bound to the filter.}$$

In addition solubility of collagen in the lower compartment of the chambers was determined by incubating chambers as for chemotaxis with collagen diluted to a concentration of 100 μg/ml in the lower compartment. After 6 h the solution in the lower compartment was diluted 1:2 in sample buffer (1 M Tris, pH 6.8, 10% SDS, 20% glycerin, 0.025% bromphenol blue, 2% β-mercaptoethanol) and electrophoresed under reducing conditions in an 8% polyacrylamide gel. Equal volumes of the original collagen solution were also electrophoresed for comparison. Following Coomassie Blue staining, the intensity of protein stain was compared.

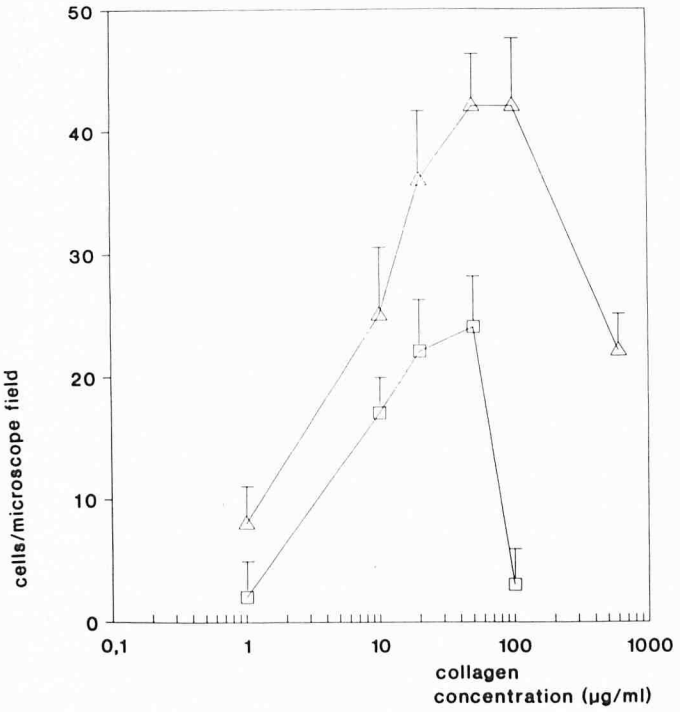


Figure 1. Chemotactic response of HaCaT cells to native triple helical (△) and denatured (□) collagen type I. Cell motility was assessed using modified Boyden chambers. Increasing concentrations of collagen were diluted in DMEM and added to the lower compartment and polycarbonate filters were placed above. After adding 2×10^5 cells to the upper compartment, chambers were incubated at 37°C for a period of 6 h. Data represent the mean of triplicate determinations for a single representative experiment of migrated cells per microscope field ($\bar{X} \pm$ SEM). All experiments were repeated three times.

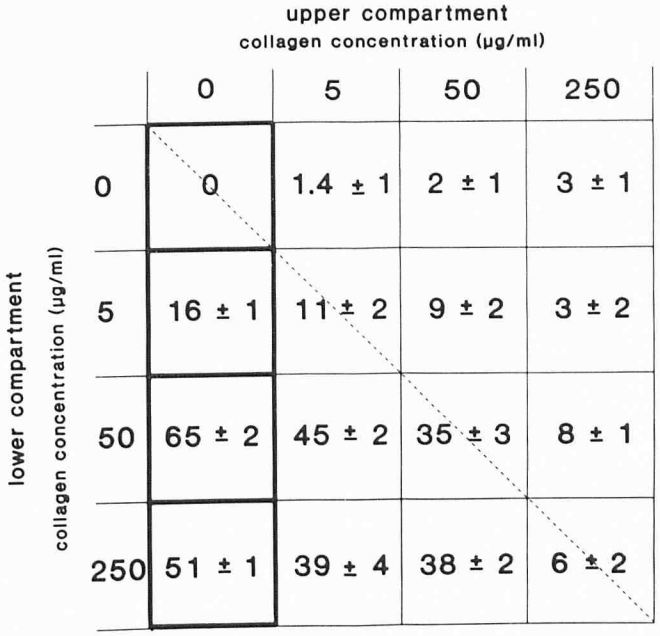


Figure 2. Checkerboard analysis of the migratory response of HaCaT cells to collagen. Varying concentrations of soluble collagen type I were added to the upper chamber together with the cells or to the lower chamber, as indicated. The migratory response is expressed as the mean of triplicate determinations of migrated cells per microscope field ($\bar{X} \pm$ SEM). *Vertical boldfaced data* indicate the migration observed to a maximal positive gradient. Data along the *diagonal* represent enhanced random migration that occurs in the absence of an established gradient.

Digestion of Collagen Type I with Bacterial Collagenase: Native unlabeled and ^{125}I-labeled collagen (100 µg/ml) (specific activity 4×10^{-2} µCi /mole) was dialyzed against DMEM without supplements. To 3 ml of each of these solutions 40 µg of clostridial collagenase in 50 µl DMEM (Worthington, CLSPA, Biochrom, Berlin, FRG) was added. Native collagen at a higher concentration (5 mg/ml) was digested with collagenase (same charge as mentioned above) in parallel. Digestion was carried out at 37°C, pH 7.5, for various periods of time. Collagenase was inactivated by adding 50 µl of 0.1 M EDTA or 85 µl of 1 M EDTA, respectively. The digests of unlabeled collagen at initial concentration of 100 µg/ml was used for migration experiments, whereas the digests of unlabeled collagen at initial concentrations of 5 mg/ml were subjected to SDS-PAGE [35] using an 8% polyacrylamide gel. Gels were stained with Coomassie Blue and photographed. The ^{125}I-labeled digests were precipitated by adding two volumes of 40% ammoniumsulfate and centrifuged at 10,000 rpm for 10 min. The precipitates were redisolved in 0.05% acetic acid and, in parallel to the supernatants, radioactivity was quantitated in a gamma scintillation counter.

Preparation of Denatured Collagen: Denatured collagen was prepared by heating native collagen in acetic acid for 30 min to 45°C and then dialyzing in the cold overnight against DMEM [5].

Attachment Assay: Attachment of HaCaT cells to the filter in the presence of different concentrations of various antibodies was assessed. Dilutions of collagen as indicated were added to the lower compartment of the chamber. Filters were overlayed and 2×10^5 cells/ml were dispensed into the upper compartment. In another set of experiments attachment of HaCaT cells to filters pre-coated with various concentrations of collagen on the lower side of the filter was assessed in the presence of different concentrations of various antibodies. The assay was stopped after 2 h. The filters were washed twice cautiously with PBS and the attached cells were then fixed, stained, and counted as described for the chemotaxis assay.

Preincubation of HaCaT Cells with RGD Peptides: HaCaT cells were pre-incubated with RGD peptides (Boehringer Mannheim, FRG) in serum-free medium at a concentration of 25 µg/ml and 250 µg/ml for 1 h at 25°C. In the presence of the same concentrations of RGD peptides in the upper compartments of the Boyden chambers chemotaxis was measured in response to 100 µg/ml of soluble collagen or 25 µg/ml of soluble fibronectin, respectively.

RESULTS

Chemotactic Migration of HaCaT Cells to Collagen Type I

HaCaT cells migrated in a concentration-dependent manner to soluble collagen type I (Fig 1). The migratory response was found to be biphasic. Maximal stimulation occured at 50–100 µg/ml but significant migration occured already at 10 µg/ml. High concentrations elicited less than maximal or no migration. Checkerboard analysis [1] was performed to assess the directed (chemotactic) versus the random (chemokinetic) nature of cellular migration. Chambers were assembled with different ratios of collagen type I below and above the filter. The values along the diagonal indicate random migration to equal concentrations of soluble collagen added to both compartments of the chamber, whereas the values below the diagonal reflect responses to a positive gradient. The data presented in Fig 2 show maximal induction of random migration at 50 µg/ml collagen (35 ± 3 cells/microscope field) but this was clearly less than the chemotactic response of 65 ± 2 cells/microscope field obtained with a concentration gradient of 0–50 µg/ml collagen. Diffusion of ^{125}I-labeled collagen across the filter during a 6-h incubation period was about 15% (data not shown) indicating that the concentration gradient of collagen was maintained. In order to confirm that collagen remained in solution during the assay, two sets of

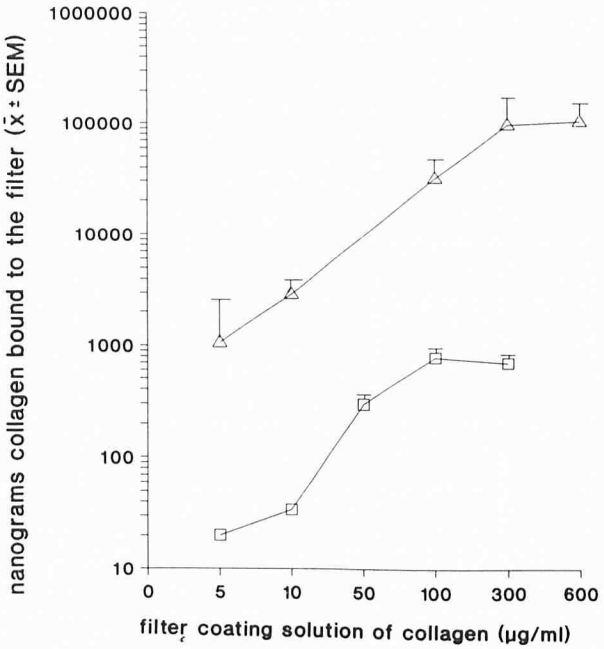
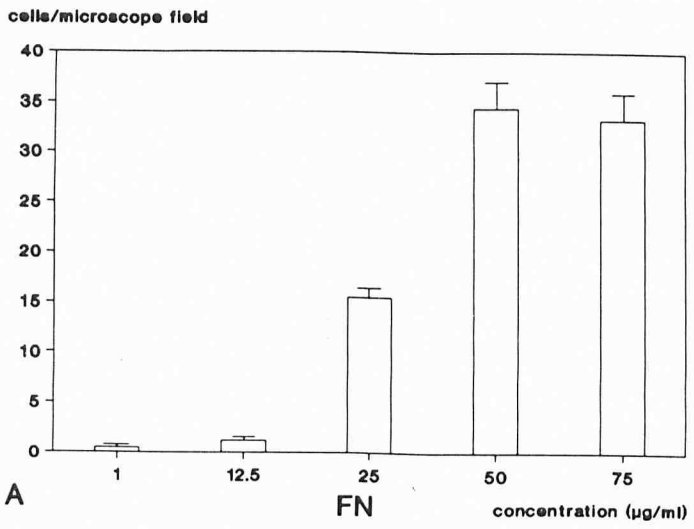


Figure 3. Binding of ^{125}I-labeled collagen to the untreated (□) or to the gelatin-pre-treated polycarbonate filters (△). The indicated concentrations of ^{125}I-labeled collagen were incubated in assembled chambers in the absence of cells for 6 h. Determinations of radioactivity bound to the filters were done in triplicate and are represented as the mean of bound collagen ($\bar{X} \pm$ SEM).

control experiments were performed. First, samples of the collagen solution (100 µg/ml) that had been incubated in the Boyden chambers were assayed by SDS-PAGE in comparison with samples of the original solution. As judged by Coomassie Blue staining, the content of collagen type I remained the same in the samples before and after incubation indicating that virtually all collagen type I stayed in solution during the period of the experiment (data not shown). Secondly, ^{125}I-labeled collagen type I was introduced into the lower compartment and incubated for 6 h. Thereafter, 85–90% of the initial radioactivity was still in the liquid phase, whereas 10–15% of the radioactivity was detected on the chamber walls. Only 0.05–0.3% of the radioactivity was associated with the filter indicating that only small amounts of collagen bind to the filter (Fig 3, *lower graph*). In comparison to collagen type I, fibronectin and laminin were also used as chemoattractants. Although fibronectin was chemoattractive for HaCaT cells, laminin was not (Fig 4*A, B*).

Haptotactic Migration of HaCaT Cells to Collagen To determine whether haptotaxis was involved in the directional migration of HaCaT cells, collagen type I precoated filters were used in the migration assay. Prior to coating with collagen type I (1–600 µg/ml), filters were pretreated with gelatin, as detailed elsewhere [33]. This was necessary, because only gelatin-pretreated filters were able to bind high amounts of collagen (Fig 2, *upper graph*), whereas filters that had not been pretreated showed only low collagen type I binding. The highest rate of migration occurred when the filter was coated on the distal side only. The migratory response was reduced by 30% when both the upper and the lower side of the filter had been coated (Fig 5). Also, fibronectin induced haptotaxis of HaCaT cells (Table I), whereas coating of the filters with laminin had no effect. In control experiments, haptotaxis was studied with filters precoated with BSA (1–600 µg/ml) but no significant haptotactic migration was observed (data not shown).

Responsiveness of HaCaT Cells to Collagenase-digested Collagen and to Heat-Treated Denatured Collagen Bacterial collagenase is known to completely digest the triple helix of the collagen type I molecule. When collagen that had been preincubated with bacterial collagenase was introduced into the assays, cell migration was totally abolished. As shown in Fig 6*A* loss of chemoattraction of collagen type I depended on the duration of collagenase digestion. A decrease in the chemoattractive potential was already noted after 30 min of collagenase digestion and was almost complete after 24 h. The digestion of collagen type I was also documented by SDS-PAGE (Fig 6*B*). We also digested ^{125}I-labeled collagen with bacterial collagenase. After 16 h of incubation, 95% of the radioactivity was detected within the supernatant of the 40% ammonium sulfate precipitate, indicating that the digestion of collagen was almost complete (data not shown). Conversion of the native triple helical to the random coil conformation of the constituent alpha chains of collagen can be accomplished by heat treatment



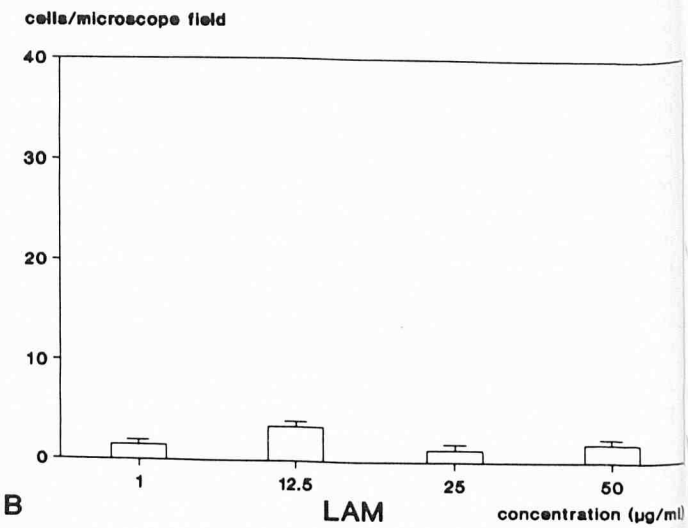


Figure 4. Concentration-dependent chemotactic response of HaCaT to fibronectin *(A)* and laminin *(B)*. Cell motility was assessed using modified Boyden chambers as described in Fig 1. Data represent the mean of triplicate determinations for a single representative experiment of migrated cells per microscope field ($\bar{X} \pm$ SEM). All experiments were repeated three times.

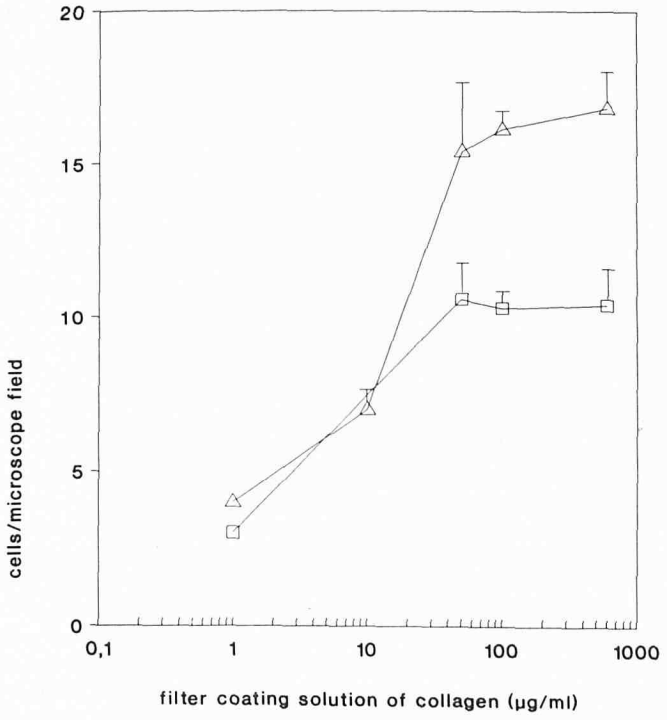


Figure 5. Haptotactic response of HaCaT cells to collagen type I. Cell motility was analyzed using modified Boyden chambers. Filters were pretreated with gelatin and then coated on only the lower (distal) (△) or on both sides (□) of the filter with increasing concentrations of collagen. After washing and air drying, filters were used in the migration assay in the absence of soluble collagen. Data represent the mean of triplicate determination for a single representative experiment of migrated cells per microscope field ($\bar{X} \pm$ SEM). All experiments were repeated three times.

[5]. Figure 1 *(lower graph)* shows that this reduced but not totally abolished the chemo- (Fig 1) and hepatotactic properties (data not shown) of collagen type I for HaCaT cells.

Preincubation of HaCaT Cells with RGD Peptides Following preincubation of HaCaT cells with RGD tripeptides, no inhibition of chemotaxis to collagen type I was detected, whereas that to fibronectin was almost completely inhibited (Fig 7). A maximal inhibition was already noticed at a concentration of 25 µg/ml.

Table I. Fibronectin-Induced Haptotaxis of HaCaT Cells[a]

Protein Concentration (µg/ml)	Side of the Filter Coated	Migrated Cells/ Microscope Field
Fibronectin		
5	Distal	5 ± 1
5	Both	2 ± 1
25	Distal	8 ± 2
25	Both	3 ± 1
50	Distal	16 ± 2
50	Both	2 ± 1
Laminin		
5	Distal	4 ± 1
5	Both	3 ± 1
25	Distal	3 ± 2
25	Both	3 ± 2
50	Distal	4 ± 2
50	Both	3 ± 1

[a] Filters were pre-coated on the distal side only or on both sides with various concentrations of fibronectin or laminin. Following washing and air drying, filters were used in the migration assay in the absence of soluble attractants. Data represent the mean number of migrated cells per microscope field (×200) ± SEM. Determinations were done in triplicate.

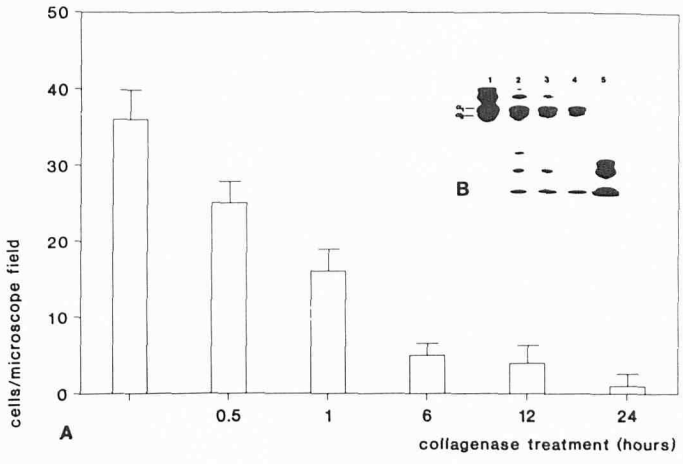


Figure 6. *A)* Chemotactic response of HaCaT cells to collagen type I (100 µg/ml) after collagenase treatment as a function of incubation time (h) compared to untreated collagen. Native collagen was digested with clostridial collagenase as detailed in *Materials and Methods.* Following digestion for various periods of time, samples were assessed for their chemotactic activity using the Boyden chamber assay as described in Fig 1. Data represent the mean number of migrated cells per microscope field ($\bar{X} \pm$ SEM). Determinations were done in triplicate. *B)* Following digestion of collagen with collagenase for various periods of time, samples were subjected to polyacrylamide gel electrophoresis *(lanes 2–5). Lane 1:* native collagen (5 mg/ml). *Lanes 2–5:* native collagen digested with collagenase for 0.5 h *(lane 2),* for 1 h *(lane 3),* for 12 h *(lane 4),* and for 24 h *(lane 5).*

Specific Involvement of VLA-2 in Collagen Type I–Induced Migration We then investigated whether one of the three known collagen receptors of the integrin gene family is involved in the chemotactic response to collagen type I.

Radioimmunoprecipitation studies of [^{35}S]-methionine–labeled monolayer cultures showed that VLA-2 and VLA-3 but not VLA-1

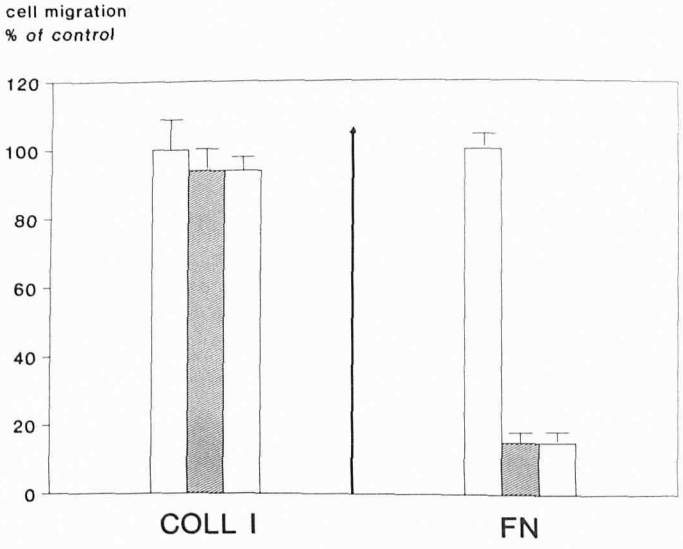


Figure 7. Effect of RGD peptides on type I collagen- and fibronectin-stimulated motility. HaCaT cells were preincubated with RGD peptides at 25 µg/ml *(hatched bars)* or 250 µg/ml *(dotted bars)* for 1 h at 25°C before migration assays. Cells preincubated with DMEM were tested as controls *(open bars).*

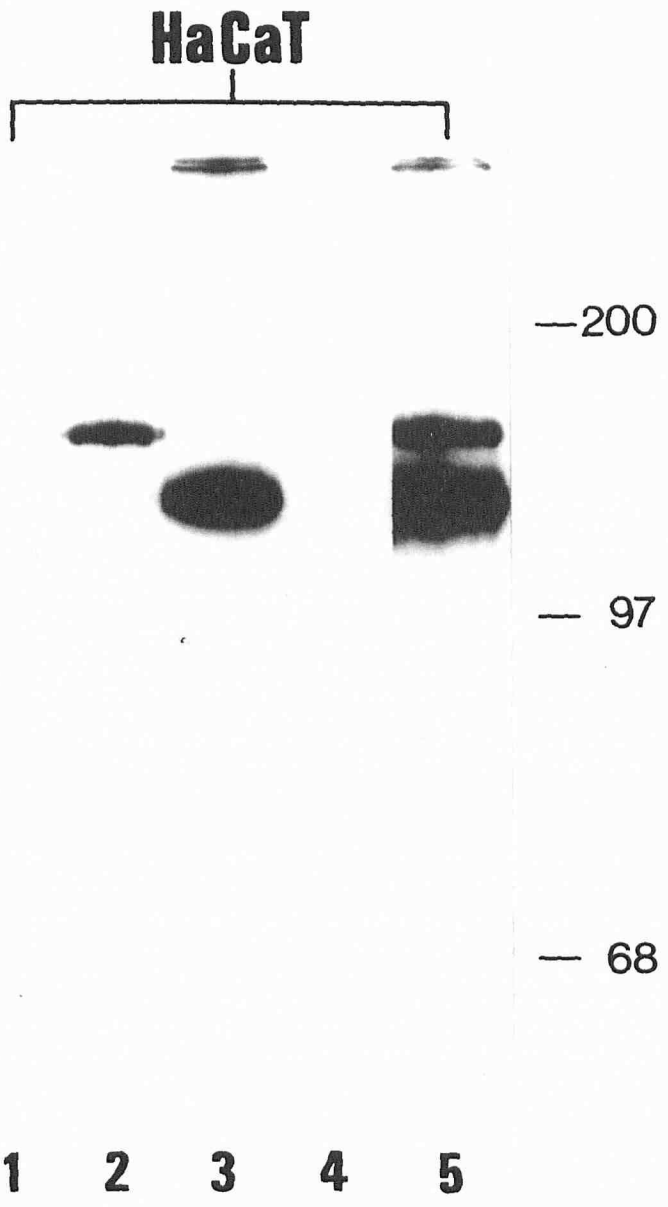


Figure 8. Synthesis of VLA-receptors in HaCaT cells. Con A–bound fractions of NP-40 cell lysates from [^{35}S]-methionine–labeled subconfluent cultures were analyzed by immunoprecipitation and SDS-PAGE. The fluorogramm shows immunoprecipitates obtained with different MoAb: *(lane 1)* Ts 2/7, anti α_1-chain, VLA-1; *(lane 2)* 5E8, anti α_2-chain, VLA-2; *(lane 3)* J 143, anti α_3-chain, VLA-3; *(lane 4)* normal mouse serum, control; *(lane 5)* 4B4, anti β_1-chain, all VLA receptors.

are synthesized by HaCaT cells (Fig 8). As a next step, we tried to inhibit chemotaxis by preincubation of HaCaT cells with function-blocking MoAb directed to the α_2-, α_3-, α_6-, and β_1-chains of the VLA-receptors (Fig 9).

MoAb 5E8 (anti α_2), AIIB2, and 4B4 (both anti β_1) completely inhibited migration of HaCaT cells in response to collagen type I at concentrations of 0.1 μg/ml, 1 : 10 dilutions, and 1 μg/ml, respectively. MoAb P1B5 (anti α_3) and GoH$_3$ (anti α_6) showed no inhibitory effect. Other MoAb directed against unrelated cell-surface molecules (Lv230, W6/32, F19) did not affect migration to collagen. This strongly suggested that VLA-2 ($\alpha_2\beta_1$) is the integrin receptor essential for migration. Control experiments revealed that the MoAb did not interfere with cell attachment to the upper side of the uncoated filters in the presence of soluble collagen in the lower compartment (Fig 10) as well as with collagen-coated filters (data not shown).

DISCUSSION

In this report we have demonstrated collagen type I–induced migration of the immortalized human HaCaT cells serving as a model for skin keratinocytes. This migration was mediated by the VLA-2/collagen receptor and could be abolished by specific blocking antibodies. Checkerboard analysis indicated that the response of HaCaT cells to collagen type I was complex. Cell migration was most stimulated when soluble collagen was added to the lower compartment of the Boyden chamber only (directed migration). Migration increased in a dose-dependent fashion to a maximal level of 50–100 μg/ml, which was followed by a decline at higher concentrations. This may at least partly be attributed to fibril formation of native collagen at higher concentrations so that the actual concentration of collagen molecules in solution is very low. Similar dose-response relations have been reported for other cell types and attractants [1,34,36]. Enhanced migration was also detected in response to soluble collagen added simultaneously on both sides of the filters (increased random migration). Random migration, however, was clearly less than directed migration.

There are several lines of evidence that the directed migration of HaCaT cells to collagen type I is predominantly due to a fluid-phase gradient (chemotaxis) and, to a small degree, to substratum-bound collagen (haptotaxis). This was supported by the finding that only small amounts of ^{125}I-collagen bound to the untreated filters under the assay conditions used for chemotaxis. In contrast, gelatin–pretreated filters as used for evaluation of haptotaxis bound significantly higher amounts of collagen. However, even under these conditions, significant migration was only detected at coating concentrations of 100 μg/ml of collagen. Moreover, migration was only slightly increased when filters were coated on the lower side of the filter only as compared to both sides thus indicating a certain but minor degree of haptotaxis. Previously, Woodley and co-workers [12] had shown enhanced keratinocyte motility in response to collagen that was allowed to attach to coverslips. However, the authors tested only one collagen concentration (30 μg/ml) and the type of migration was not further characterized.

The specific effect of collagen type I on HaCaT cell migration was further substantiated by time-dependent inhibiton of keratinocyte migration following disintegration of the triple helix into small fragments by bacterial collagenase. Transition of the triple helical to the random coil formation of the collagen α-chains was associated with some, but not complete, loss of the attractive properties. The reason for the difference in attractive potency of native triple-helical collagen and the constituent collagen α-chains are not apparent from our data. Perhaps the ordered triple-helical confirguration of collagen type I favors binding of the molecule to its corresponding receptors on the surface of HaCaT cells. A strong conformation dependence has already previously been postulated for collagen type I–mediated fibroblast attraction [5,9].

To evaluate whether HaCaT cell migration could be modulated by other matrix proteins, the migratory response of HaCaT cells to fibronectin and laminin was tested. HaCaT cell migration was induced in resoponse to filter-bound fibronectin as well as to soluble fibronectin, whereas no migration occured to filter-bound laminin or soluble laminin. This corresponds to earlier published data that fibronectin stimulates, whereas laminin inhibits, motility of human keratinocytes in primary culture [12].

Receptor-ligand interactions have been proposed to mediate directed leukocyte migration [37–40]. Recently, three collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$) have been identified [16,41–43] that belong to the integrin superfamily [13,14], but so far have only been characterized to mediate cell adhesion to collagen [16,41,42,44–46].

There is accumulating evidence that integrins serve as transmembrane linkers between their extracellular ligands and the cytoskeleton [47,48]. In this capacity integrins have been presumed to promote cellular migration [14,48]; however, it has not yet been

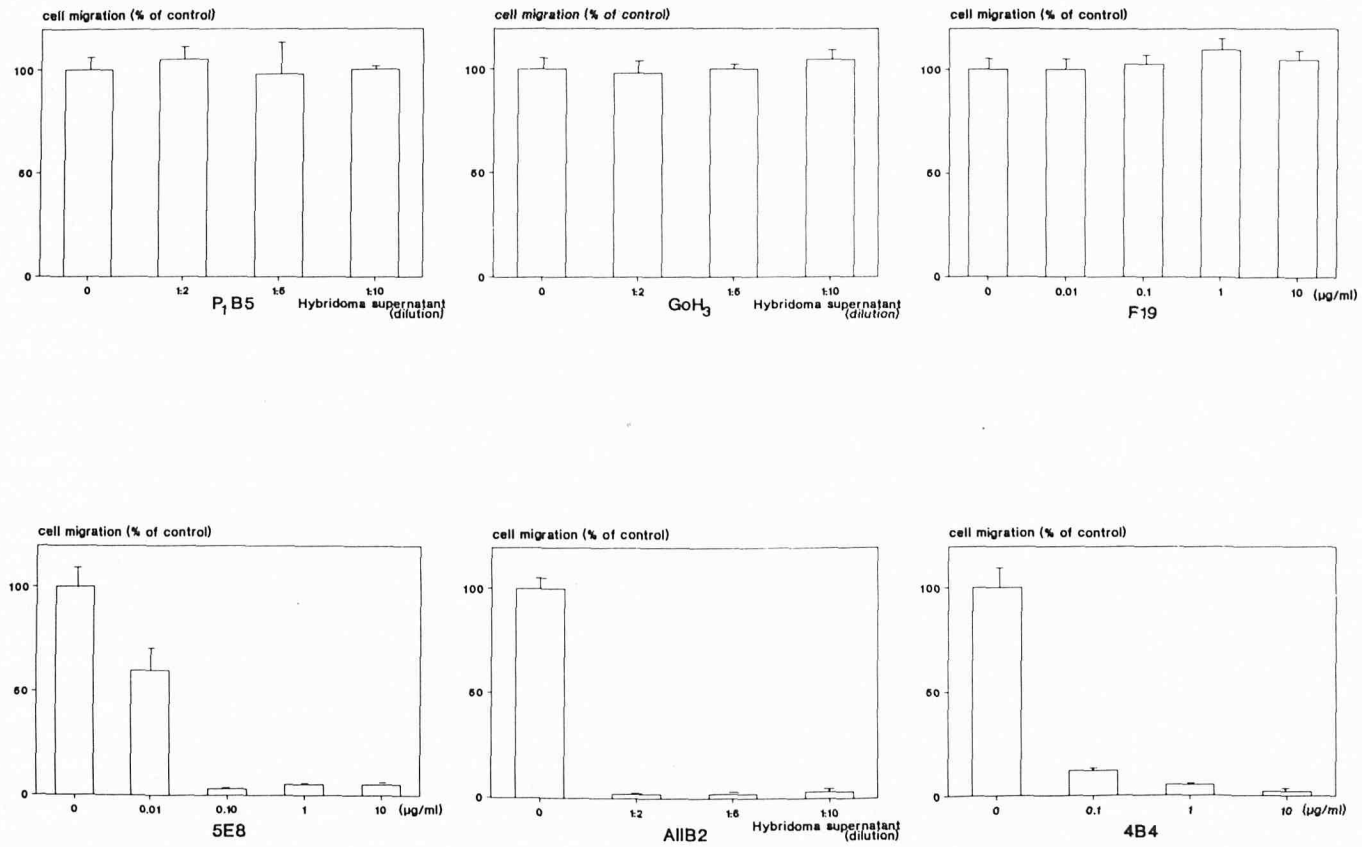


Figure 9. Effect of various antibodies on migration of HaCaT cells to collagen type I. Prior to the migration assay, cells were incubated with the indicated concentrations of various antibodies for 10 min in serum-free medium (P_1B5, anti α_3-chain; GoH_3, anti α_6-chain; F19, antibody directed against an unrelated cell surface protein; 5E8, anti α_2-chain; AIIB2, anti β_1-chain; 4B4, anti β_1-chain). Cell migration to collagen type I (100 μg/ml) was then assessed as decribed in Fig 1. Cell migration was expressed as percent of the untreated control. The average of triplicate assays is presented.

conclusively shown whether integrin receptors are involved in cellular migration to defined ligands. We here provide evidence that the $\alpha_2\beta_1$ collagen receptor mediates keratinocyte migration to collagen type I. First, we analyzed which of these collagen receptors are expressed by HaCaT cells. Immunoprecipitating from lysates of [^{35}S]-methionine-labeled HaCaT cells indicated that the receptors $\alpha_2\beta_1$ and $\alpha_3\beta_1$ but not $\alpha_1\beta_1$ were synthesized.

Inhibition studies with RGD peptides did not result in a decreased migration to collagen type I, whereas migration of HaCaT cells to fibronectin was almost abolished. These data suggest that the putative receptor involved in attraction of HaCaT cells to collagen type I is—contrary to the fibronectin receptor (VLA-5) [6,14,49]—independent of the RGD peptide. This is in line with previously published data for the $\alpha_2\beta_1$ receptor, which binds to the α_1(I)-CB3 peptide of collagen not containing an RGD sequence [50]. The strongest line of evidence that the VLA-2 receptor mediates epidermal cell migration to collagen comes from inhibition studies using antibodies that selectively block the α_2 or β_1 subunit of the VLA-2 receptor. Both antibodies dose-dependently blocked the chemotaxis of HaCaT cells to collagen type I. As obvious from attachment studies this effect is not due to decreased attachment of cells to the filters. Because the migration of HaCaT cells could be completely blocked by the 5E8 antibody, which is directed against the α_2 subunit, we concluded that the VLA-2 receptor and not the co-expressed VLA-3 receptor mediates collagen type I-induced migration of HaCaT cells. MoAb 5E8 has previously been used to inhibit the growth of lung tumor cell lines in vitro [51]. In addition, it selectively inhibits the adhesion of platelets to collagen type I (M.E. Hemler, Boston, MA, personal communication). As controls MoAb directed to unrelated cell-surface glycoproteins and function-blocking MoAb recognizing the α_6-[29] and α_3-chain [27] of integrins have been used. None of them altered the migration of HaCaT cells to collagen type I, thus underlining the specifity of the effect of the MoAb 5E8.

This study was performed with an immortalized human keratinocyte cell line (HaCaT) established from adult human skin, which is remarkably stable in its phenotype and which under confluent conditions reveals a keratin pattern similar to that of normal keratinocytes [17,19,20]. Also, in regard to the response to external signals, which modulate differentiation (e.g., calcium levels, retinoids) [18], HaCaT cells resemble normal keratinocytes [52]. HaCaT cells maintain full epidermal differentiation capacity when transplanted onto nude mice where they form a rather normal epidermis with expression and typical localization of specific keratins and other markers (*involucrin, filaggrin*) [17,19]. Most important for this study, however, is that these cells express the $\alpha_2\beta_1$ collagen receptor at similar high levels as primary keratinocyte cultures [46] (Klein et al, unpublished). Although the HaCaT cells share many features with normal keratinocytes it remains to be confirmed whether migration of normal human keratinocytes in culture, and more important in situ, in response to collagen type I [12] is also mediated by the $\alpha_2\beta_1$ receptor. Therefore, the biologic significance of our findings with HaCaT cells for the behavior of keratinocytes in vivo still bears some uncertainty but its possible mechanism and role in tissue repair of the skin has important implications for future work. It is tempting to envisage a minimal sequence of events. During cutaneous wound healing, epidermal cells that are physiologically separated from the dermis by the basement membrane are in direct contract to the newly synthesized collagen type I [53]. Because epidermal reconstitution by proliferation, differentiation, and migration parallels de novo synthesis of collagen type I and other extracellular matrix proteins it is reasonable to assume that collagens are directly

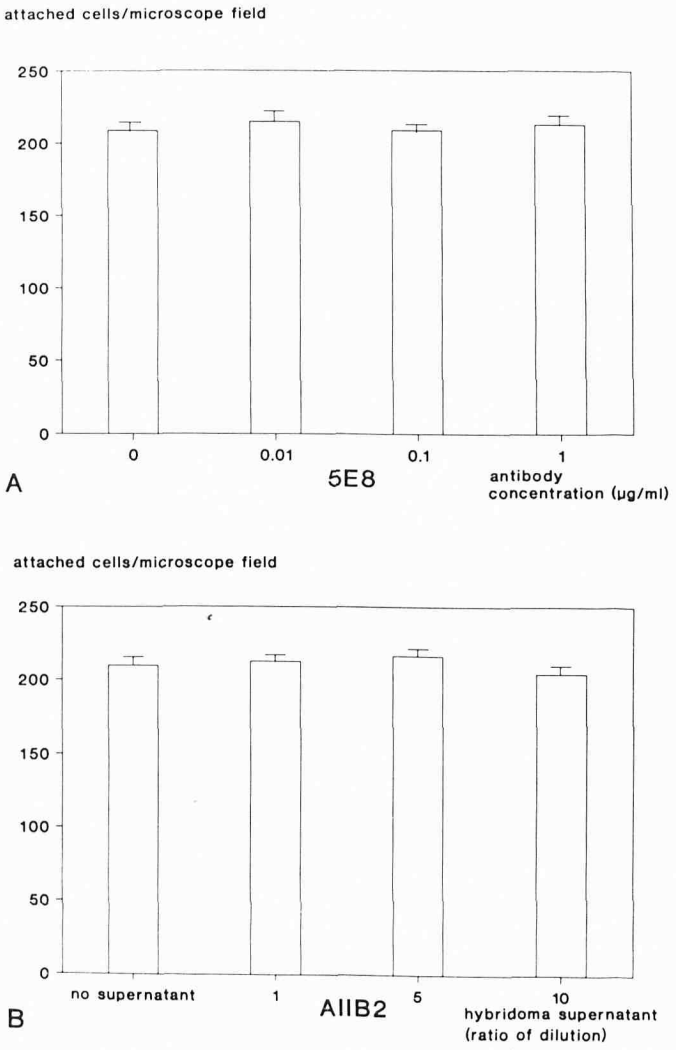


Figure 10. Attachment of HaCaT cells to the polycarbonate filter in the presence to different concentrations of the 5E8 antibody *(A)* and different dilutions of the hybridoma supernatant containing the AIIB2 antibody *(B)* in the upper compartment of the Boyden chamber. Dilutions of the antibodies as indicated were added to the upper compartment of the chamber together with 2×10^5 cells/ml. The lower compartment of the Boyden chamber contained soluble collagen (100 µg/ml). After 2 h, filters were carefully washed twice with PBS and the attached cells were then fixed, stained, and counted. Data represent the mean of duplicate determinations ($\bar{X} \pm$ SEM).

involved in the attraction of epidermal cells, as has already been shown for fibronectin [54]. Interstitial collagen type I and III have been found to be sequentially synthesized following initial production of fibronectin during cutaneous restoration [55] thus possibly perpetuating cellular migration required for epidermal reconstitution.

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